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elegans Heterochronic Gene *lin-28* after Translation Initiation

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The heterochronic gene *lin-28* of the nematode *Caenorhabditis elegans* controls the relative timing of diverse developmental events during the animal's larval stages. *lin-28* is stage-specifically regulated by two genetic circuits: negatively by the 22-nt RNA *lin-4* and positively by the heterochronic gene *lin-14*. Here, we show that *lin-28* is repressed during normal development by a mechanism that acts on its mRNA after translation initiation. We provide evidence that *lin-14* inhibits a negative regulation that is independent of the *lin-4* RNA and involves the gene *daf-12*, which encodes a nuclear hormone receptor. The *lin-4*-independent repression does not affect the initiation of translation on the *lin-28* mRNA, and like the *lin-4*-mediated repression, acts through the gene's 3′-untranlsated region. In addition, we find that *lin-4* is not sufficient to cause repression of *lin-28* if the *lin-4*-independent circuit is inhibited. Therefore, the *lin-4*-independent circuit likely contributes substantially to the down-regulation of *lin-28* that occurs during normal development. The role of *lin-4* may be to initiate or potentiate the *lin-4*-independent circuit. We speculate that a parallel *lin-4*-independent regulatory mechanism regulates the expression of *lin-14*. © 2002 Elsevier Science (USA)

***Key Words:* *C. elegans*; heterochronic genes; *lin-4*; *lin-14*; *lin-28*; polyribosomes; small temporal RNA; microRNA; translational regulation; translation run-off.**

INTRODUCTION

The relative timing of postembryonic developmental events is explicitly regulated in the nematode *Caenorhabditis elegans*. Regulators encoded by the heterochronic genes act in tissues throughout the animal to coordinate the succession of cell fates within lineages (reviewed in Slack and Ruvkun, 1997; Ambros, 2000). At each larval stage, a subset of these regulators acts as a switch between alternative choices of cell fates. The activation and repression of the heterochronic genes at specific times are critical components of the developmental timing mechanism. If a heterochronic gene is mutant, development is either precocious, skipping stage-specific events, or retarded, reiterating stage-specific events in subsequent stages (Ambros and Horvitz, 1984). Several heterochronic genes encode novel regulatory molecules. Among these are two very small RNAs that act as gene-specific repressors at the level of mRNA translation (the terms small temporal RNA, stRNA, and microRNA, miRNA, have been coined to refer to them; Lee *et al.*, 1993; Reinhart *et al.*, 2000; Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001).

The heterochronic gene *lin-4* encodes a 22-nt RNA that acts in the first larval stage (L1) to repress two other heterochronic genes, *lin-14* and *lin-28* (Lee *et al.*, 1993; Wightman *et al.*, 1993; Moss *et al.*, 1997). *lin-4* is not expressed until the mid- to late-L1 stage, when it begins the down-regulation of *lin-14* and *lin-28* (Feinbaum and Ambros, 1999). *lin-14* and *lin-28* encode nuclear and cytoplasmic proteins, respectively, that likely control downstream effectors of specific developmental processes (Ruvkun and Giusto, 1989; Moss *et al.*, 1997). Both *lin-14* and *lin-28* must be active in the L1 and repressed later for developmental events to occur in the appropriate succession. If *lin-4* is mutated and fails to function, *lin-14* and *lin-28* continue to be expressed at late stages and retarded development results (Arasu *et al.*, 1991; Moss *et al.*, 1997). The 3′ UTR of each of these genes contains sequence elements necessary for its repression, and it is believed that *lin-4* acts by basepairing with these elements.

The stage-specific repression of *lin-14* has been shown to

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occur posttranscriptionally—the level of the LIN-14 protein decreases without a corresponding change in the level of its mRNA (Wightman *et al.*, 1993). The mechanism of this regulation was investigated by Olsen and Ambros (1999) and was found to occur at a point after translation initiation. Although unusual, other cases of postinitiation translational regulation have been characterized (e.g., Berry *et al.*, 1990; Ch'ng *et al.*, 1990; Kaspar and Gehrke 1994; Chapman and Walter, 1997; Clark *et al.*, 2000; Rüegsegger *et al.*, 2001). The *lin-4* RNA can be found associated with ribosomes, and it was suggested that it functions by affecting the translation process itself or the fate of the newly synthesized polypeptides (Olsen and Ambros, 1999).

In addition to being repressed by *lin-4*, *lin-14* and *lin-28* positively regulate each other. Arasu *et al.* (1991) observed that a stage-specific repression of *lin-14* may occur without *lin-4* if *lin-28* activity is reduced by mutation. Likewise, stage-specific repression of a *lin-28* reporter gene occurs in a *lin-4* mutant if *lin-14* activity is compromised (Moss *et al.*, 1997). Although these findings describe a positive feedback loop between *lin-14* and *lin-28*, they also suggest the existence of a *lin-4*-independent repression that acts on each gene in the other's absence. Like *lin-4*-mediated repression, this genetic circuit is temporally regulated; there is no evidence of it acting early in the L1, but rather it acts later, apparently simultaneously with *lin-4*. To date, no regulators involved in the *lin-4*-independent regulation, other than *lin-14* and *lin-28* themselves, are known.

Here, we investigate the nature of the stage-specific repression of *lin-28*. We find that, like *lin-14*, *lin-28* is posttranscriptionally repressed at a point after translation initiation. We provide evidence that the positive regulation of *lin-28* by *lin-14* involves the inhibition of a repression that is independent of *lin-4* and acts through the 3′ UTR of *lin-28*. We also find that this *lin-4*-independent repression affects *lin-28* expression after translation initiation. Significantly, we provide evidence that *lin-4* is not sufficient to repress *lin-28* without the *lin-4*-independent circuit. We suggest that the *lin-4*-independent repression makes a significant contribution to the down-regulation of *lin-28*. We speculate that a similar combination of regulatory circuits acts on *lin-14*.

MATERIALS AND METHODS

Nematode Strains and Culturing

Strains were cultured according to standard techniques (Lewis and Fleming, 1995). Wild-type and nontemperature-sensitive strains were maintained at 20°C. VT573 and MT1388, both temperature-sensitive strains, were maintained at 20°C and grown at 25°C for experiments. The strains used were N2 wildtype, DR441 *lin-14(n179ts)*, DR721 *lin-4(e912)*, ME1 *lin-28(ga54); lin-46(ma164)*, ME65 *daf-12(rh61)*, MT355 *lin-14(n355sd)*, MT1388 *lin-14(n355n679ts)*, and VT573 *lin-4(e912); lin-14(n179ts)*. ME1 is the *lin-28(0)* strain used in the experiments shown in Figs. 1 and 5. ME65 was derived from a strain kindly provided by Adam Antebi (Max-Planck-Institut, Berlin).

Synchronized cultures of nematodes were prepared by growing populations in suspension in complete S medium or on NGM agar plates (Lewis and Fleming, 1995). *Escherichia coli* JM83 was used as food. Embryos were harvested from gravid adults by treatment with alkaline hypochlorite and synchronized by hatching them overnight in M9 medium (Lewis and Fleming, 1995). Synchronous newly hatched L1 larvae were then grown with food to the required stage of development. Large-scale cultures of N2 were grown in suspension at 20°C for 12 h, when the majority of worms are in the mid-L1 stage, and at 30 h, when the majority are in the L3 stage. The staging of each culture was verified by inspection by DIC microscopy, examining the gonad and hypodermal development. VT573 was grown at 25°C for 9 and 21 h to achieve the same staging. Other mutants were grown on solid medium until the majority of the animals were in L3, as determined by inspection. Because of the unavoidable asynchrony that accumulates in populations grown for many hours, L3 stage was chosen as the late stage in these experiments to reduce the number of early-stage worms in the culture as much as possible. At the end of the growth period, the larvae were separated from *E. coli* and debris by floatation on 35% sucrose. The animals were then washed in water, drop-frozen in liquid nitrogen, and stored at −70°C.

Sucrose Gradient Analysis

Frozen pellets of synchronized larvae were ground to a powder with a cold mortar and pestle. Ground worms were resuspended in an equal volume of Buffer U: 200 mM Tris–Cl, pH 8.5, 50 mM KCl, 25 mM $MgCl_2$, 2 mM EGTA, 500 μg/ml heparin, 2% polyoxyethylene 10-tridecyl ether (PTE), 0.75% sodium deoxycholate (Davies and Abe, 1995). Extracts were clarified at 17,000 rpm (27,000*g*) in a Sorvall ST-micro rotor for 12 min at 4°C. Extracts were normalized by RNA content by absorbance at 260 nm. RNase-protection assays were used to compare the amount of mRNA present in the supernatant and the pellet after clarification, and they established that the bulk of the mRNA was present in the supernatant (data not shown).

Equivalent amounts of early- and late-stage cytoplasmic extracts (approximately 40 A260 units) were applied to the top of a 20–60% sucrose gradient and centrifuged at 40,000 rpm in a Beckman SW41 rotor for 2 h at 4°C. Fractions were collected by using an ISCO density gradient fractionation system monitored by absorbance at 254 nm. Absorbance tracings were collected digitally. Approximately 20 0.5-ml fractions were collected per gradient. Fractions were ethanol precipitated, resuspended in polysome extraction buffer (20 mM Tris–Cl, pH 7.4, 2.5 mM EDTA, 100 mM NaCl, 1% SDS), phenol extracted, and reprecipitated, and mRNA levels were measured by RNase-protection assay.

Translation Run-Off

Translation run-off was performed by using purified *C. elegans* polyribosomes in a rabbit reticulocyte lysate after methods previously described (Vayda, 1995). Cytoplasmic extracts of late-stage (30 h) larvae were prepared as described above. The extracts were applied to 50–60% sucrose cushions, and polysomes were pelleted by centrifugation in an SW55 rotor at 50,000 rpm for 2 h. Pelleted polysomes were resuspended in RNase-free water, and 0.75 A260 units in 20 μl were combined with 33 μl of nuclease-treated rabbit reticulocyte lysate (Promega, L4960) and 2 μl of amino acid mix (Promega). The run-off assay was performed on ice or at 25 or 30°C with and without 0.3 mg/ml cycloheximide for 45 min. The entire

mixture was fractionated on 20–60% sucrose gradients, and the absorbance profile at 254 nm was taken during fractionation. The monosomal peak increased linearly with increasing incubation time and leveled off after 1–2 h (data not shown). Reinitiation of translation appeared to be negligible. A concentration series showed that the run-off was sensitive to cycloheximide down to 2.6 μg/ml (9 μM), and the effect of cycloheximide was 80% of the maximum at 11 μg/ml (data not shown).

Antisera and Immunoblots

Antisera were raised in rabbits to full-length his-tagged recombinant LIN-28 protein expressed in *E. coli*. Dilutions of 1:2000 of the sera were used. Immunoblots were performed by using BioRad mini electrophoresis and transfer apparatuses (BioRad), PVDF transfer membrane (NEN), and developed with a chemiluminescence kit (NEN). Secondary antibody was HRP-conjugated donkey anti-rabbit Ig (Amersham). As a gel loading control, anti-actin monoclonal antibody AC-15 (Sigma) and HRP-conjugated sheep anti-mouse Ig (Amersham) were used. Cytoplasmic extracts of staged populations were prepared as described above for sucrose gradients and were normalized by protein concentration. A protein-null allele of *lin-28*, *lin-28(ga54)*, was used as a negative control (Moss *et al.*, 1997).

RNase-Protection Assays

RNase-protection assays were performed by using RPA II kit from Ambion. All probes were gel-purified prior to use. The probes were specific to *lin-28*, *lin-14*, and the eIF4A gene (Wightman *et al.*, 1991; Roussell and Bennett, 1992; Moss *et al.*, 1997). The probes were transcribed *in vitro* from plasmid subclones. Each probe was complementary to part of an intron and an exon, so that fragments protected by mRNA are shorter than the undigested probes. The digested radioactive products of the assays were separated on polyacrylamide gels. The gels were dried and then analyzed with a Fuji phosphorimager. Quantitation of the signals was performed by using the MacBAS v2.2 software. The bands representing each digested probe were selected with a box across the gel image. The background signal was subtracted graphically. The signal in each lane was then divided by the total signal for that probe across the gradient, yielding the relative signal intensity in each fraction of the gradient. Many lanes include some signals due to undigested probe which may be protected by folding of the probe or by protection by DNA.

Transgenic Reporter Assay

pVT#218, which contains a translational fusion of GFP to the *lin-28* genomic clone (Moss *et al.*, 1997) was modified by replacing the 3′ UTR and downstream sequences of *lin-28* with the 3′ UTR and downstream sequences from the *unc-54* gene from plasmid pPD95.02 (gift from A. Fire, Carnegie Institution). The construct was introduced into DR441 *lin-14(n179ts)* by microinjection along with cosmids RIp16 and C33C3. Transgenic animals were identified based on expression of GFP in the L1 stage and then examined in later stages. Transgenic adults were photographed by DIC and fluorescence microscopy.

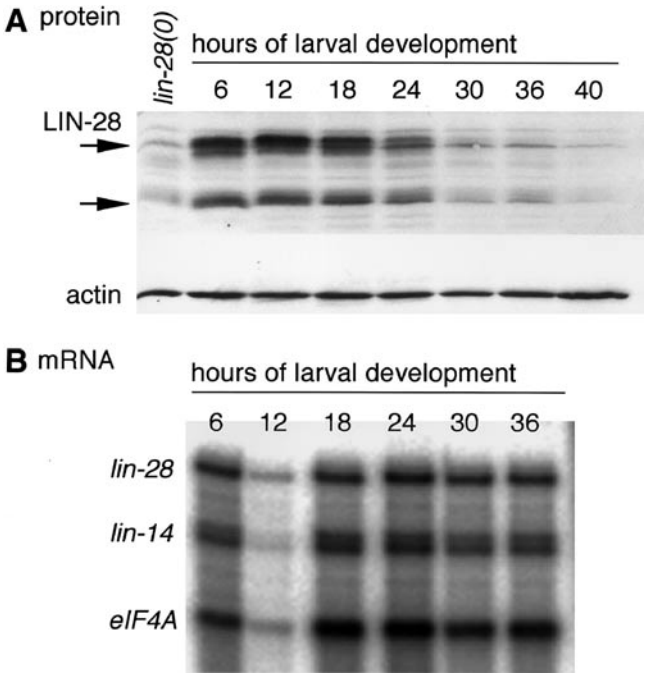

FIG. 1. The abundance of LIN-28 protein and *lin-28* mRNA during larval development. (A) Top, Immunoblot using anti-LIN-28 antisera of extracts of a population of wild-type animals harvested at 6-h intervals from the beginning of larval development. Corresponding stages: 6, 12, and 18 h, L1; 24 h, L2; 30 h, L2/L3; 36 h, L3, 40 h, L3/L4. Bottom, Immunoblot using anti-actin antibody as a loading control. (B) RNase-protection assay of *lin-28*, *lin-14*, and eIF4A mRNA levels in extracts harvested at 6-h intervals of larval development. The probes protect a portion of the coding region of each gene and do not correspond to the size of the full-length mRNAs. Although the 12-h lane is underloaded, the ratios of the mRNA levels are approximately the same as in the other lanes (data not shown).

RESULTS

lin-28 Is Posttranscriptionally Repressed

The stage-specific repression of *lin-28* was observed previously by using a *lin-28:GFP* reporter transgene (Moss *et al.*, 1997). To assess the regulation of the endogenous LIN-28 protein, anti-LIN-28 antisera was developed and used in immunoblots of preparations of staged larvae (Fig. 1A). The antisera, which was raised against full-length recombinant protein, detects at least three LIN-28 isoforms: a doublet at approximately 36 kDa and a single band at 28 kDa, which are the products of alternative splicing and posttranslational modification (E.G.M. and L.T., unpublished). These species are not detected in a strain that carries a null allele of *lin-28* [Fig. 1A; *lin-28(0)*]. Extracts were prepared from larvae harvested at 6-h intervals after beginning L1 development. Samples were normalized by total protein content prior to loading, and detection of actin

with an anti-actin antibody was performed as a loading control. All forms of LIN-28 were abundant during the L1 stage (Fig. 1A; 6–18 h) and decreased to their lowest levels by the L3 stage (30–36 h). The timing of this decrease is similar to that seen in individual animals using the *lin-28: GFP* reporter transgene (Moss *et al.*, 1997). Titration experiments revealed that the decrease in LIN-28 protein level from 12 to 30 h of larval development was between 10- and 20-fold (data not shown). Others have shown that the level of LIN-14 protein decreases to the same degree over a similar time period (Wightman *et al.*, 1993; Olsen and Ambros, 1999). These observations are consistent with genetic results that indicate *lin-28* is active early and repressed later to allow a transition from L2 to L3 development. The change in endogenous LIN-28 protein levels is consistent with a down-regulation of expression that reflects a temporal regulation of *lin-28* gene activity.

To determine whether the decrease in LIN-28 protein reflected a decrease in mRNA level, RNase protection assays were performed on total RNA extracted from larvae harvested at the same 6-h intervals (Fig. 1B). In addition to *lin-28,* two other genes were included in the analysis: *lin-14* and the gene encoding eIF4A. *lin-14* mRNA was previously shown to change little through larval development (Wightman *et al.*, 1993; Olsen and Ambros, 1999). The eIF4A expression is continuous and serves as a control for total mRNA level (Roussell and Bennett, 1992; Olsen and Ambros, 1999). The samples were normalized at the start of the assays by total RNA content. The level of *lin-28* mRNA was constant over the entire period examined, from the start of the L1 (6 h) to the early- to mid-L3 (36 h). As expected, both *lin-14* and eIF4A mRNAs were also constant during this period. To ensure that the protein levels and mRNA levels at each time point could be compared, the total RNA levels of each extract used in immunoblots were measured and were found to be equivalent (data not shown). Therefore, the drop in LIN-28 protein level cannot be accounted for by a change in *lin-28* mRNA level, suggesting *lin-28* is regulated posttranscriptionally.

Down-Regulation of lin-28 Causes No Change in the Association of lin-28 mRNA with Polyribosomes

To determine whether the down-regulation of LIN-28 protein level was due to an effect on translation, we examined the polyribosome association of the *lin-28* mRNA. It was previously shown that *lin-14* is repressed posttranscriptionally by a mechanism that does not affect the association of the *lin-14* mRNA with polysomes (Olsen and Ambros, 1999). Because *lin-28* is repressed in parallel, we sought to determine whether the same phenomenon occurs. Cytoplasmic extracts were prepared from early- (12-h) and late-stage (30-h) synchronized populations of larvae and sedimented in 20–60% sucrose gradients. Fractions of the gradients were collected, RNA was isolated, and the mRNA levels of both *lin-14* and *lin-28* were measured by RNase-protection assay (Fig. 2A). The eIF4A mRNA was again included as a control. The polysomal (P), monosomal (M), and submonosomal (S) regions of the gradient were identified from the absorbance profile at 254 nm taken during fractionation, which is a measure of total RNA in each fraction (data not shown). At both early and late stages, the *lin-28* mRNA was in the polysomal fractions, as were the *lin-14* and eIF4A mRNAs. To determine whether the mRNAs were in fact associated with ribosomes in these fractions, extracts were treated with EDTA prior to loading on the sucrose gradient to dissociate ribosomes and mRNAs. EDTA treatment resulted in a complete shift of all three mRNAs from the polysomal and monosomal fractions into the subpolysomal fractions after sedimentation (data not shown). Therefore, the *lin-28* mRNA, as well as the *lin-14* and eIF4A mRNAs were likely associated with polysomes at both early and late stages.

To quantitatively assess distributions of each mRNA in the gradients, the signal in each fraction was normalized to the total signal for that mRNA across the gradient, and the corresponding fractions from each gradient were compared (Fig. 2B). No appreciable shift of any of the mRNAs out of the polysomal fractions from early to late stages was evident. A change in polysome association of an mRNA would be expected if initiation of translation was affected. The distribution of the eIF4A mRNA was not expected to change because it is constitutively expressed. The association of the *lin-14* mRNA with polysomes also does not change, as has been shown previously (Olsen and Ambros, 1999). Here, we observed that, despite a 10- to 20-fold decrease in LIN-28 protein level, the *lin-28* mRNA was associated with polyribosomes equivalently at early and late stages. These observations suggest that *lin-28* is regulated posttranscriptionally by a mechanism that does not affect translation initiation.

The differences in amounts of each mRNA present in the subpolysomal fractions at both stages are likely to reflect the inherent translation rates of the mRNAs, where more efficiently translated mRNAs, specifically the eIF4A mRNA, are present to a lesser amount in these fractions. The presence of *lin-14* mRNA in heavier polysome fractions than *lin-28* mRNA likely reflects the fact that this mRNA is capable of loading more ribosomes due to its longer open reading frame (1.6 kb vs. 0.7 kb).

lin-14 and lin-28 mRNAs Are Associated with Translation-Competent Ribosomes

At late stages of development, the *lin-14* and *lin-28* mRNAs may be associated with stalled ribosomes or they may be actively translated, even though their proteins do not accumulate. To address these possibilities, we determined whether the ribosomes bound to the mRNAs late in development are indeed capable of translation. Polyribosomes were purified from late-stage larvae, and their ability to continue translation was determined by a translation run-off assay (Vayda, 1995). The purified polysomes were

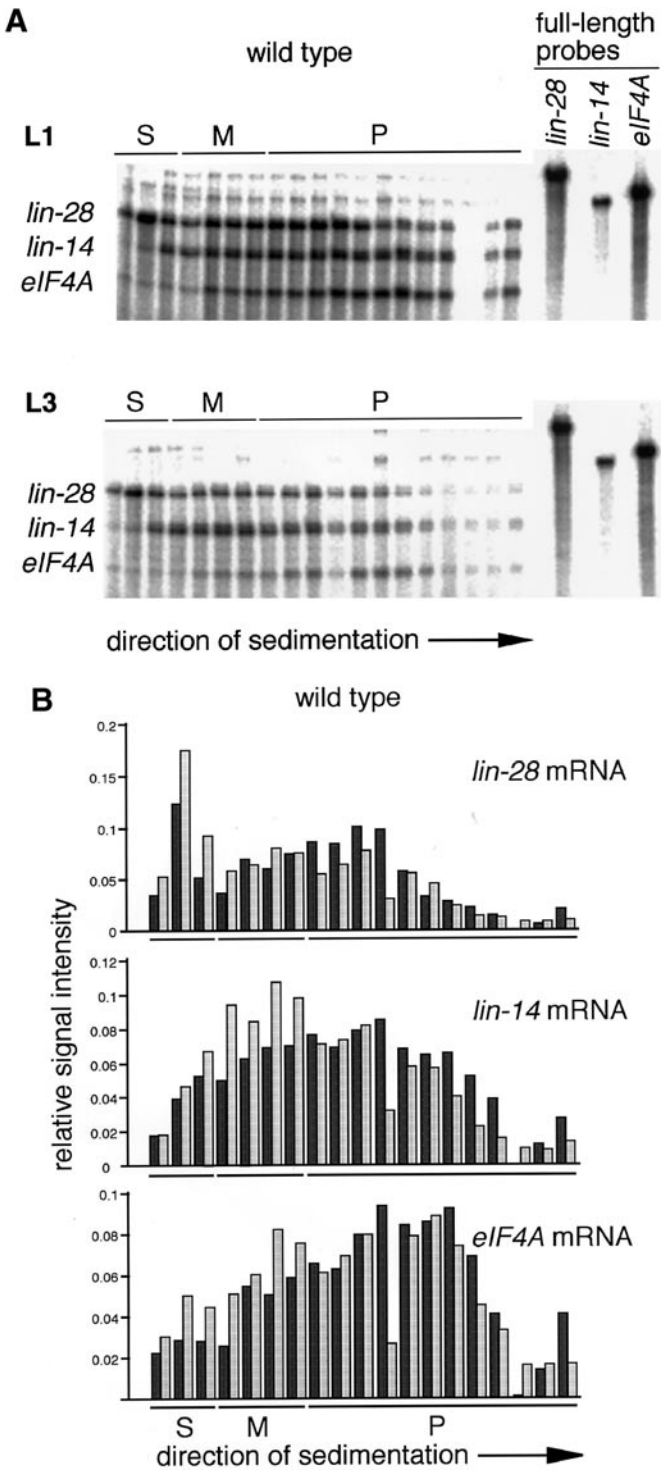

FIG. 2. Comparison of sucrose gradient distributions of mRNAs from early- and late-stage wild-type *C. elegans*. (A) RNase-protection assays showing *lin-28, lin-14,* and eIF4A mRNA levels in each fraction of a 20-60% sucrose gradient. Extracts were made from larvae grown 12 (L1) and 30 h (L3) at 20°C. Lanes with full-length probes are to the right. Undigested probe may be due to protection by DNA. S, submonosomal fractions; M, monosomal fractions; P, polysomal fractions. (B) Histograms of the signal intensity in each fraction relative to the total signal for that mRNA across the gradient. Dark bars, L1 stage. Light bars, L3 stage.

incubated with a rabbit reticulocyte lysate with or without cycloheximide, a specific inhibitor of translation elongation, or placed on ice. After incubation, the material was fractionated on 20-60% sucrose gradients, and the distribution of the *lin-14* and *lin-28* mRNAs was measured by RNase-protection assay. If the mRNAs were not translated, then they would still be in the polysomal fractions of the gradient. If they were translated, the mRNAs would be found in the subpolysomal fractions.

The absorbance tracing taken during the fractionation showed the effect of the three different incubation conditions on the *C. elegans* polyribosomes in general (Fig. 3A). When incubation was carried out with cycloheximide or on ice, the normal polysome profile was maintained: to the right of the monosomal peak is the absorbance due to polysomal material with individual peaks visible (Fig. 3A, center and right). Polyribosomes are not present in the nuclease-treated reticulocyte lysate, so the absorbance in the polysomal region of the gradient reflects *C. elegans* material only (data not shown). When incubation was carried out without cycloheximide, the polysomes dissociated concomitant with an increase in the monosomal peak (Fig. 3A, left). Because the polysomes were stable in the presence of cycloheximide, we infer that their dissociation during the incubation without cycloheximide was due to translation run-off and not to cleavage of the mRNA.

To determine whether the ribosomes bound to *lin-14* and *lin-28* mRNAs were translation-competent, the distributions of these mRNAs in the gradient fractions were measured. Adjacent fractions were pooled to increase the mRNA concentration, and RNase-protection assays were performed by using *lin-28, lin-14,* and eIF4A mRNA probes (Fig. 3B). All three mRNA species were observed in polysomal (P) fractions when the material was incubated on ice or with cycloheximide, as expected (Figs. 3B and 3C, center and right). However, when the late-stage *C. elegans* polysomes were incubated with the reticulocyte lysate without cycloheximide, the three mRNAs were shifted to lighter fractions (Figs. 3B and 3C, left). Although some degradation of mRNA occured in the reactions incubated at the higher temperature, the same overall shift in the distributions of the mRNAs was observed in repetitions of the experiment (data not shown). The sensitivity of the shift to cycloheximide indicates that it was the result of translation elongation. Although we currently cannot assess whether the repression of *lin-14* and *lin-28* is preserved in the translation run-off reaction, we have confirmed that translation run-off produces new polypeptides (data not shown). These data suggest that the *lin-14* and *lin-28* mRNAs are actively translated at late stages.

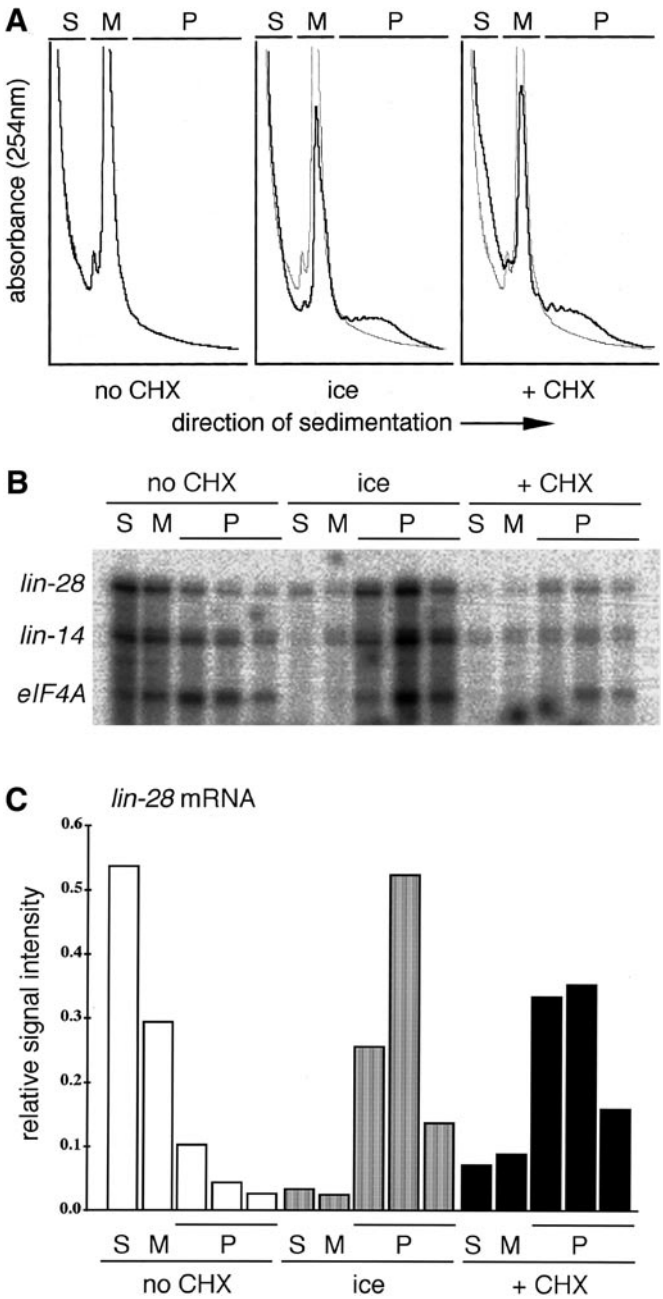

FIG. 3. Translation run-off of *C. elegans* polyribosomes from late-stage extracts. (A) Absorbance (254 nm) profiles taken during fractionation on a 20-60% sucrose gradient after incubation of purified *C. elegans* polyribosomes in a rabbit reticulocyte lysate without cycloheximide (no CHX), on ice, or with cycloheximide (+ CHX). The highest peaks of the tracings have been omitted for clarity. The tracing in the no CHX reaction is overlayed in a lighter line onto the other two tracings for comparison. (B) RNase-protection assays showing *lin-28*, *lin-14*, eIF4A mRNA levels in pooled adjacent fractions of the gradients shown in (A). S, submonosomal fractions; M, monosomal fractions; P, polysomal fractions. (C) Histogram of the *lin-28* mRNA signal intensity the RNase protection assays in (B) relative to the total signal across each gradient.

lin-4-Independent Repression of lin-28 Also Does Not Change the Association of the lin-28 mRNA with Polyribosomes

lin-28 is stage-specifically repressed independently of *lin-4* if *lin-14* activity is reduced, indicating the existence of a *lin-4*-independent repression mechanism (Moss *et al.*, 1997; see below). Although this repression is genetically distinct from the action of *lin-4*, its affect on *lin-28* expression may or may not occur by the same mechanism. To compare the effects of these two genetic circuits, we determined whether the *lin-4*-independent repression of *lin-28* affects the association of *lin-28* mRNA with polyribosomes.

A strain of the genotype *lin-4(e912); lin-14(n179ts)* carries a deletion of the *lin-4* gene and a temperature-sensitive missense allele of *lin-14* (Ambros and Horvitz, 1987; Lee *et al.*, 1993; Reinhart and Ruvkun, 2001). Cytoplasmic extracts of early- and late-stage synchronized populations of these animals grown at the restrictive temperature were fractionated on 20-60% sucrose gradients, and RNase-protection assays were performed on the fractions (Fig. 4A). As in wild type, the *lin-28* mRNA was associated with polyribosomes at early and late stages. Normalizing the signal in each lane to the total signal for that mRNA across the gradient showed that the polyribosome distribution of the *lin-28* mRNA was unchanged from early to late stages (Fig. 4B). The distributions of *lin-14* and eIF4A mRNAs were also unchanged during larval development. Thus, it appeared that a reduction of *lin-14* activity at a late stage of development did not affect translation initiation of the *lin-28* mRNA.

The *lin-4*-independent repression of *lin-28* in the *lin-4(e912); lin-14(n179ts)* strain was previously observed by using a *lin-28:GFP* reporter transgene (Moss *et al.*, 1997). To verify that the endogenous protein was down-regulated without *lin-4* present, the level of LIN-28 protein at early and late stages in this strain was assessed by immunoblot (Fig. 5, lanes 2 and 3). As in wild type, LIN-28 protein level decreased substantially from early to late stages in this strain. This was in contrast to the high level of LIN-28 seen at a late stage in a *lin-4* mutant with a wild-type *lin-14* gene (Fig. 5, lane 4). Together, these observations indicate that the *lin-4*-independent repression of *lin-28* also occurs post-transcriptionally by a mechanism that does not affect translation initiation, and in that way is indistinguishable from the repression seen normally when *lin-4* is present.

lin-4 Is Not Sufficient to Fully Repress lin-28

The similarity between the *lin-4*-independent repression of *lin-28* and that seen in wild type led us to evaluate the relative contributions of *lin-4* and the *lin-4*-independent circuit to *lin-28* regulation. As mentioned above, we observed that endogenous LIN-28 protein was stage-specifically down-regulated in the absence of *lin-4* in *lin-4(e912); lin-14(n179ts)* animals. Reciprocally, we wished to determine whether LIN-28 was down-regulated by *lin-4* in the absence of *lin-4*-independent repression.

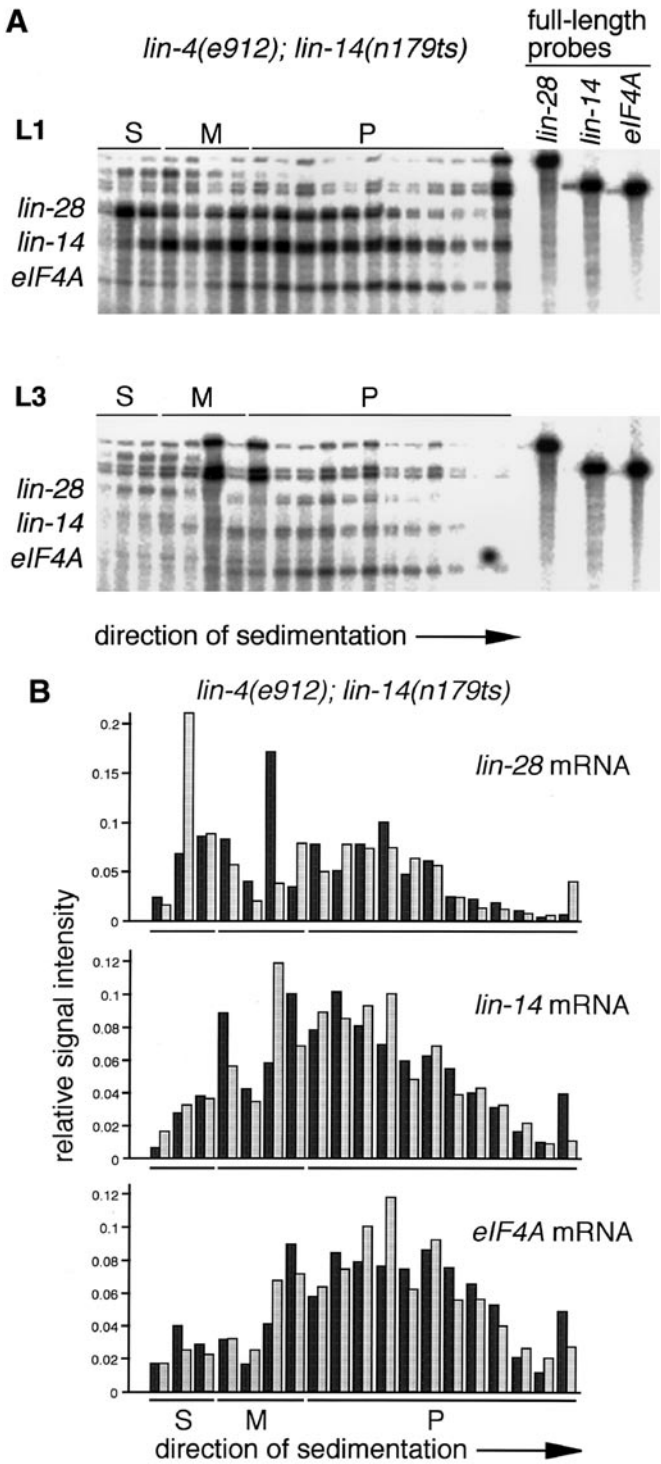

FIG. 4. Comparison of sucrose gradient distributions of mRNAs from early- and late-stage *lin-4; lin-14* mutant *C. elegans* extracts. (A) RNase-protection assays showing *lin-28, lin-14,* and eIF4A mRNA levels in each fraction of a 20-60% sucrose gradient. Extracts were made from larvae grown 9 (L1) and 21 h (L3) at 25°C. Lanes with full-length probes are to the right. Undigested probe may be due to protection by DNA. S, submonosomal fractions; M, monosomal fractions; P, polysomal fractions. (B) Histograms of the signal intensity in each fraction relative to the total signal for that mRNA across the gradient. Dark bars, L1 stage. Light bars, L3 stage.

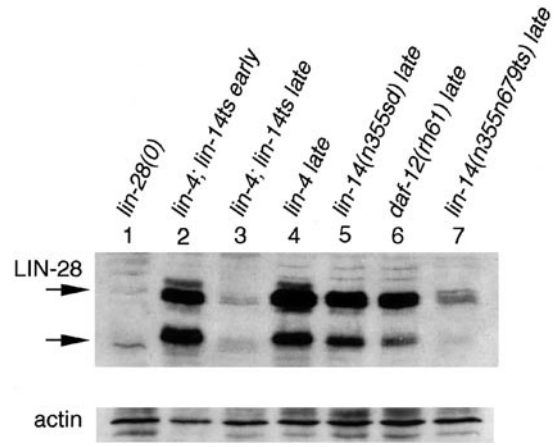

FIG. 5. LIN-28 protein abundance in *C. elegans* mutants at early and late stages of larval development. Top, Immunoblot using anti-LIN-28 antisera of extracts of populations of *C. elegans* mutants harvested at early and late stages of larval development. Bottom, Immunoblot using anti-actin antibody as a loading control.

The *lin-4*-independent repression of *lin-28* occurs only if *lin-14* activity is reduced (Fig. 5, lanes 3 and 4; Moss *et al.*, 1997). *lin-14(n355sd)* is a mutation in which *lin-4*-responsive elements of the *lin-14* 3′ UTR of *lin-14* are missing, and consequently, *lin-14* expression is not down-regulated in this strain (Ruvkun and Giusto, 1989; Arasu *et al.*, 1991; Wightman *et al.*, 1991). We have determined that this strain has wild-type *lin-4* gene that is expressed (data not shown). Therefore, in *lin-14(n355sd)* animals, it is expected that *lin-4* is active, whereas the *lin-4*-independent circuit is not. Late-stage extracts of *lin-14(n355sd)* animals were examined by immunoblot with anti-LIN-28 antisera (Fig. 5, lane 5). LIN-28 protein was found to be abundant, and the level was comparable to that in a *lin-4(e912)* animals at similar stages (Fig. 5, lane 4). A strain with a second-site mutation in *lin-14* that reduces its activity, *lin-14(n355n679ts),* shows a much lower LIN-28 protein level, demonstrating that LIN-28 can be down-regulated in this strain if *lin-14* activity is reduced (Fig. 5, lane 7). Therefore, *lin-28* expression was not fully repressed if *lin-14* activity is elevated, despite the presence of *lin-4*.

To further support this finding, we examined LIN-28 expression in another strain in which *lin-4* is normal but the *lin-4*-independent repression is likely to be compromised. *daf-12(rh61)* displays a retarded heterochronic phe-

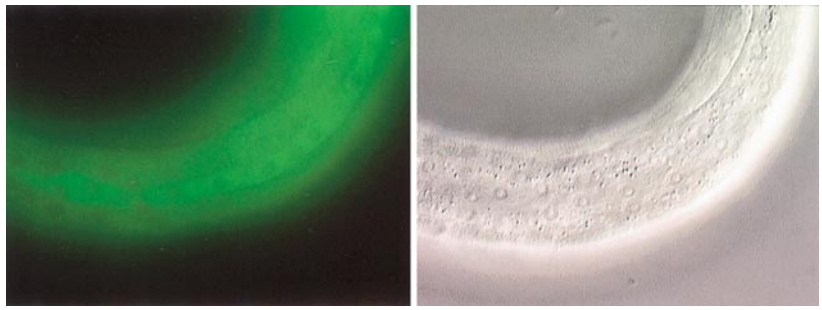

FIG. 6. *lin-4*-independent repression of *lin-28* requires the 3′ UTR. Micrographs of a animal of the genotype *lin-14(n179ts); lin-28:GFP: unc-54 3′ UTR*. The animal as determined to be an adult by the extent of somatic gonad and germline development. Left, Fluorescence micrograph showing GFP expression and extra seam cells. Right, DIC image of the same field.

notype and has been shown to be epistatic to a *lin-14* null mutation and hypostatic to a *lin-28* null mutation (Antebi *et al.*, 1998). A prediction based on these observations is that a retarded *daf-12* mutation would cause continued LIN-28 protein synthesis late in development, even in the presence of *lin-4*, regardless of *lin-14* activity. The level of LIN-28 protein in *daf-12(rh61)* animals was assessed by immunoblotting (Fig. 5, lane 6). As was observed for *lin-14(n355sd)*, the LIN-28 protein level is abundant late in development. Taken together, these results indicate that *lin-4* on its own is not sufficient to fully down-regulate *lin-28* expression, and suggest that the *lin-4*-independent regulatory circuit makes a significant contribution to the down-regulation of *lin-28* during normal development.

lin-4-Independent Repression of lin-28 Requires Its 3′ UTR

The *lin-4*-independent repression mechanism may act during translation, involving the *lin-28* mRNA, or on the LIN-28 protein after it is liberated from the ribosome. To help distinguish between these possibilities, we asked whether the *lin-4*-independent repression, like the *lin-4*-mediated repression, requires the 3′ UTR. It was previously shown that a *lin-28:GFP* transgene is repressed in the *lin-4(e912); lin-14(n179ts)* strain, demonstrating the activity of the *lin-4*-independent repression mechanism (Moss *et al.*, 1997). A variant of this reporter was constructed in which the 3′ UTR of *lin-28* was substituted with that of *unc-54*. The modified reporter was introduced into *lin-14(n179ts)* animals to test for expression of *lin-28:GFP* at late stages when *lin-14* activity is reduced. Despite the presence of a wild-type *lin-4* gene and reduced *lin-14* activity, the *lin-28:GFP:unc-54* 3′ UTR transgene showed strong expression at late stages and caused supernumary seam cell divisions, a retarded developmental phenotype (Fig. 6). This observation is consistent with the conclusion that *lin-4*-independent repression requires the 3′ UTR of *lin-28*, and therefore is not likely to be a posttranslational mechanism acting on the LIN-28 protein itself.

DISCUSSION

The stage-specific repression of the heterochronic gene *lin-28* is critical for the normal timing of *C. elegans* larval development. Here, we show that *lin-28* is regulated post-transcriptionally by two genetic circuits, one involving the 22-nt RNA *lin-4* and one that is independent of *lin-4*. Both of these circuits appear to act through the 3′ UTR of *lin-28* and affect *lin-28* expression after translation initiation. Furthermore, we find that *lin-4* is not sufficient to cause repression of *lin-28* if the *lin-4*-independent circuit is inhibited. Therefore, the *lin-4*-independent circuit likely contributes substantially to the down-regulation seen during normal development. The role of the *lin-4* RNA may be to initiate or potentiate the down-regulation of *lin-28*. None of the specific molecular components of the *lin-4*-independent repression mechanism are currently known; however, we found that the gene *daf-12* encodes a candidate for such a factor.

The lin-4-Independent Regulatory Circuits

Figure 7 depicts the *lin-4*-independent repression of *lin-28* in the context of its interactions with *lin-4* and *lin-14*. This model is based on previous genetic and molecular data, as well as the work presented here (Ambros and Horvitz, 1987; Ambros, 1989; Arasu *et al.*, 1991; Moss *et al.*, 1997).

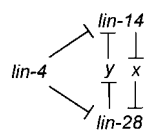

FIG. 7. The heterochronic gene regulatory heirarchy that governs early larval development. Depicted are the regulatory relationships among *lin-4*, *lin-14*, and *lin-28*, and two unidentified regulators that are responsible for the *lin-4*-independent repression of *lin-14* and *lin-28*. All regulation of *lin-14* and *lin-28* is posttranscriptional, and like *lin-4*, "*x*" acts through the 3′ UTR of *lin-28*.

Formally, *lin-14* positively regulates *lin-28* late in development; if *lin-14* activity is reduced, *lin-28* is repressed. In the case of *lin-28,* we have provided evidence that if 3′ UTR sequences of *lin-28* are replaced by 3′ UTR sequences of a heterologous gene then a reduction of *lin-14* activity does not cause down-regulation of *lin-28*. This observation implies that sequences in the 3′ UTR of *lin-28* confer negative regulation which can be inhibited by *lin-14* activity. The 3′ UTR sequences may inhibit translation directly or mediate the binding of a repressive factor. We have chosen to depict the formal positive regulation of *lin-28* by *lin-14,* as a two-step negative regulation involving an unidentified regulator "*x*".

Arasu *et al.* (1991) similarly showed a requirement of *lin-28* activity for the expression of *lin-14* late in development. This requirement is independent of the *lin-4* RNA and sequences in the 3′ UTR of *lin-14* that contain five of the seven predicted *lin-4*-complementary elements. This finding has suggested the presence of a *lin-4*-independent repression acting on *lin-14* that is inhibited by *lin-28* (Reinhart and Ruvkun, 2001). Therefore, the formal positive regulation of *lin-14* by *lin-28* may also be depicted by a two-step negative regulation (Fig. 7). Although we have not addressed this issue directly using a *lin-28* mutant, we observed that when *lin-28* expression is down-regulated in the *lin-4(e912); lin-14(n179ts)* strain, the *lin-14* mRNA remains abundant and associated with polyribosomes (Fig. 4). This observation indicates that, like the *lin-4*-independent repression of *lin-28,* this repression does not affect translation initiation either. However, currently no evidence for whether the specific components of the *lin-4*-independent mechanisms acting on *lin-14* and *lin-28* are the same. In our model, we assume a distinct regulator "*y*" represses *lin-14*.

Both of the *lin-4*-independent repression circuits are stage-specifically regulated. Early in the L1 stage, removing either *lin-14* or *lin-28* has no effect on the expression of the other, therefore the *lin-4*-independent circuits are not active at this time (Fig. 5; Arasu *et al.,* 1991; Moss *et al.,* 1997). They likely begin to function in the mid- to late-L1 stage, approximately the time that the *lin-4* RNA accumulates (Feinbaum and Ambros, 1999). Arasu *et al.* (1991) observed when *lin-28* is mutant, the LIN-14 protein level declines by the mid-L1 stage, slightly earlier than in wild type. Reinhart and Ruvkun (2001) found that LIN-14 protein level in a *lin-4(e912); lin-14(n360)* strain also decreases early in the L1, which they interpret as an effect of the *lin-4*-independent repression (*n360* is hypomorphic allele). Therefore, the time of action of the *lin-4*-independent repression circuits appears to roughly parallel the time of expression of *lin-4*.

We have observed that *lin-4,* under specific circumstances, is neither necessary nor sufficient to stage-specifically repress *lin-28*. Rather, the *lin-4*-independent repression can act without *lin-4* and shows the same post-initiation phenomenon as what is seen in wild-type development. These findings suggest that the *lin-4*-independent circuit contributes substantially to the regulation of *lin-28,* and may constitute the primary repressive mechanism. *lin-4,* although essential for normal development, may play a secondary role in the repression. We speculate that as the *lin-4*-independent circuits become active in the mid-L1 stage, they are immediately antagonized by the activities of *lin-14* and *lin-28*. *lin-4* may tip the balance in favor of repression by inhibiting *lin-14* and *lin-28* expression itself, or it may interfere with the inhibition of the *lin-4*-independent repression. How *lin-4* acts at the molecular level will be addressable once it is possible to characterize the action of specific components of the *lin-4*-independent repression circuits.

A Role for daf-12 in lin-28 Regulation

One candidate for a negative regulator of *lin-28* is the product of the *daf-12* gene. *daf-12* encodes a nuclear hormone receptor that has multiple roles in *C. elegans* development (Antebi *et al.,* 1998, 2000; Snow and Larson, 2000). Antebi *et al.* (1998) showed that certain alleles of *daf-12* cause a retarded phenotype. These authors provided evidence that *daf-12* acts independently of *lin-4* and *lin-14* but requires *lin-28* to affect developmental timing. They proposed that *daf-12* negatively regulates *lin-28,* a hypothesis we have confirmed. We have found that the *daf-12(rh61)* allele prevents complete repression of *lin-28* expression in late stages, suggesting that *daf-12* either is "*x*" in our model in Fig. 7, or it positively regulates a component of the *lin-4*-independent repression circuit that acts on *lin-28*. Although the DAF-12 protein is homologous to nuclear hormone receptors, it is possible that it binds to and regulates the *lin-28* mRNA directly. One nuclear receptor, human DAX-1, has been shown to be bound to mRNAs in the cytoplasm and associated with polyribosomes (Lalli *et al.,* 2000). Alternatively, *daf-12* may affect developmental timing by a process that is separate from the regulation of *lin-28* through its 3′ UTR. For example, *daf-12* activity could indirectly cause the degradation of existing LIN-28 protein, tipping the antagonism between positive and negative regulation of *lin-14* and *lin-28* in favor of repression. In this case, the direct mediator of the *lin-4*-independent regulation of *lin-28* would be another yet unidentified factor. It has been suggested that *let-7* regulates *lin-28,* although *let-7* is expressed too late in development to affect events of the L2 (Reinhart *et al.,* 2000). An exciting possibility is that "*x*" and "*y*" of Fig. 7 are also members of the newly discovered family of microRNAs that includes *lin-4* and *let-7* (Lagos-Quintana *et al.,* 2001; Lau *et al.,* 2001; Lee and Ambros, 2001).

Postinitiation Translational Regulation

There are growing numbers of examples of postinitiation regulation of translation (e.g., Berry *et al.,* 1990; Ch'ng *et al.,* 1990; Kaspar and Gehrke, 1994; Chapman and Walter,

1997; Olsen and Ambros, 1999; Clark *et al.*, 2000; Rüegsegger *et al.*, 2001). One of the best studied of these is the regulation of *nanos* in the preblastoderm embryo of *Drosophila* (Clark *et al.*, 2000). Repression of the *nanos* mRNA that is not localized to the posterior pole of the embryo is mediated by sequences in the gene's 3′ UTR (Gavis *et al.*, 1996). Clark *et al.* (2000) demonstrated by using a cell-free translation system derived from *Drosophila* embryos that the repressed *nanos* mRNA is associated with translating ribosomes. They propose that either factors bound to the 3′ UTR degrade or destabilize the nascent polypeptide in *cis*, or that these factors affect the processivity of the ribosome, causing premature termination of translation. Intriguing support for a direct role for the nascent polypeptide in the regulation comes from the finding that *bicaudal*, a gene that may affect *nanos* regulation directly, encodes the *Drosophila* homologue of the nascent-polypeptide associated complex β subunit (β-NAC; Markesich *et al.*, 2000). This protein in mammalian cells is bound to ribosomes and associates with the nascent polypeptide as it emerges (Wiedmann *et al.*, 1994; Beatrix *et al.*, 2000). If *bicaudal* is indeed directly involved in *nanos* regulation, then the nascent peptide would be implicated in the mechanism of repression. It has been estimated that the normal extent of cotranslational degradation of a nascent polypeptide may be over 50% (Turner and Varshavsky, 2000). This raises the possibility that a gene-specific repressive mechanism acting at the ribosome might tip the balance between biogenesis and degradation in favor of degradation. Our examination of the loss-of-function phenotype of the *C. elegans* β NAC homologue in RNA-interference experiments have not revealed a role for the protein in developmental timing (E.G.M., unpublished observations). Nevertheless, based on their present similarities, it appears that the molecular mechanism by which *nanos* and the heterochronic genes *lin-14* and *lin-28* are regulated have common mechanistic features.

In summary, we have provided evidence that the postinitiation repression that is associated with the action of the *lin-4* RNA appears to require the action of a *lin-4* independent regulatory mechanism comprised of yet unidentified regulators. Understanding how this small temporal RNA causes the down-regulation of the heterochronic genes will require identifying the components of the *lin-4*-independent regulatory circuits that act directly on the mRNAs and determining how they influence protein biogenesis.

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